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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/001,367	10/30/2001	Barbara A. Brewitt	20371.0004c4	3277

7590 02/22/2007

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EXAMINER	
SEHARASEYON, JEGATHEESAN	
ART UNIT	PAPER NUMBER
1647	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	02/22/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Application No. 10/001,367	Applicant(s) BREWITT, BARBARA A.	
	Examiner Jegatheesan Seharaseyon, Ph.D	Art Unit 1647	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 27 November 2006.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1,2,11,13-28 and 30-33 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 1, 2, 11, 13-28 and 30-33 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

1. This office action is in response to the amendments and remarks filed on 11/27/06. Claims 1, 2, 9-11 and 13-30 were pending. Claims 9, 10 and 29 have been cancelled. Claims 31-33 have been added. Therefore, claims 1, 2, 11, 13-28 and 30-33 are currently pending and are examined. The Office notes the incorrect claim inclusion in the previous Office Action.

2. The text of those sections of Title 35, U. S. Code not included in this action can be found in a prior Office action.

3. Any objection or rejection of record, which is not expressly repeated in this action, has been overcome by Applicant's response and withdrawn.

4. The allowability of the claims 24-28 is withdrawn.

5. The current status of claim 12 is not indicated in the claim listing.

Claim Rejections - 35 USC § 102(b) maintained

6. The rejection of claims 1, 11,and 14 under 35 USC § 102(b) as being anticipated over Antoniades et al. is maintained for reasons set forth in the Office Actions dated 8/24/04, 6/13/05 and 6/27/2006.

Applicant has amended claim 1 to remove the concentration of less than $1x10^{-6}$ M of IGF-1 limitation and added the homeopathic potencies of IGF-1. Applicant indicates that the composition of Antoniades et al. is directed to wound healing. Applicant argues that use of homeopathic potency of IGF-1 is not taught by Antoniades et al. Specifically, Applicant is arguing that there is no teaching or suggestion

whatsoever in Antoniades et al. that the compositions are prepared homeopathically to produce homeopathic potencies. It is argued that there is no description, either expressly or inherently, of homeopathic potencies, or of serial dilutions and serial successions. In addition, Applicant is arguing that no homeopathic nomenclature is used. Applicant's arguments have been fully considered but are not found to be persuasive because in the absence of a disclosure of a particular starting concentration of IGF-1 in claim 1 it is anticipated that the concentration disclosed by Antoniades et al. (IGF-1 of 500ng-1 μg) is included in the instant invention, regardless of the method used to prepare IGF-1 composition of the instant invention (The various homeopathic potencies could potentially include the concentration of IGF-1 disclosed in the instant invention). In addition, arguments relating to method of making were previously addressed in the Office Action dated of 6/27/2006 (pages 3-5), which have not been responded to by the Applicant in the instant response. Therefore, the rejection of record is maintained.

Claim Rejections - 35 USC § 112, first paragraph

7. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7a.Claims 1, 2, 11, 13-28 and 30-33 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter, which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to

make and/or use the invention. Specifically, the specification does not reasonably provide enablement for a preparation comprising a homeopathic potency of purified IGF-1 suitable for oral administration.

The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. See *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 The factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue" include, but are not limited to: (1) the breadth of the claims; (2) the nature of the invention; (3) the state of the prior art; (4) the level of one of ordinary skill; (5) the level of predictability in the art; (6) the amount of direction provided by the inventor; (7) the existence of working examples; and (8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Claims 1, 2, 11, 13-28 and 30-33 are drawn to a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration. Applicant is clearly contemplating using very dilute concentration of the purified protein (pages 7 and 18). In the absence of a starting concentration of IGF-1, there is no guidance, which provide for the concentrations of the various homeopathic potencies contemplated for oral administration following the serial dilutions and serial successions. Although, 6X, 6C, 15X, 12C, 30C, 100C, 200C and 1M (1000C) potencies are art accepted, neither specification nor prior art teaches the starting concentration of the purified IGF-1 used in the preparation. The specification as filed is insufficient to enable one skilled in the art to

practice the claimed invention without an undue amount of experimentation because there is no teaching to indicate the starting concentration of IGF-1. The specification teaches (page 7) that homeopathic medicines are used at concentrations of micrograms (10^{-6} M) and nanograms (10^{-12} M). It also indicates that in other homeopathic preparations, the dilutions exceed avogadro's number 6.023 $\times 10^{-23}$. However, there is no guidance to teach one of skilled in the art to dilute purified IGF-1 to obtain the homeopathic potency of the instant invention (starting from what concentration!). Specifically, it does not teach the starting concentration of IGF-1, which is required to obtain the various homeopathic potencies of the instant invention. Further, given the physiological serum concentration of IGF-1 is 200ng/ml (Dunger et al. U.S. Patent No. 5, 466, 670) it is unclear if administering a homeopathic potency of IGF-1 will have any clinical relevance. Specifically, the specification does not teach any methods or working examples that would indicate that a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration in any subject for treatment.

In addition, regarding oral administration for treatment, variables such as biological stability, half-life or clearance from the blood are important parameters in achieving successful therapy. Further, there is no teaching in the specification that would indicate that a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration would have any clinical effect upon the administration. In addition, one skilled in the art would not be able to predict the effects of the homeopathic potency of purified IGF-1 administered orally. The purified IGF-1 may not otherwise reach the target cell or tissue because of its inability to penetrate tissues or

cells where its activity is to be exerted, it may be absorbed by fluids, cells and tissues where it has no effect, circulation into the target area may be insufficient to carry the antagonist, and a large enough local concentration may not be established (see Pettit et al.,). The specification provides insufficient guidance with regard to these issues and provides no working examples or evidence, which would provide guidance to one skilled in the art to predict the efficacy of the claimed methods with a reasonable expectation of success. Thus, undue experimentation would be required of one skilled in the art at the time the invention was made to use a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration.

Further, there is no teaching in the specification with respect to the various pathologies associated with the various physiological disorders relating to IGF-1 caused by various etiologies. The usefulness of the compositions contemplated in the claims is tied to the usefulness of the homeopathic potency of purified IGF-1 in treating various physiological disorders. In addition, the specification and the prior art have not disclosed a role for homeopathic potency of IGF-1 in the treatment of various physiological conditions.

If one skilled in the art is not guided as to the pathology of the various physiological disorders treatable using purified IGF-1, then the skilled artisan is also not guided as to how to use a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration. Since, there is inadequate guidance as to the nature of the invention, it is merely an invitation to the artisan to use the current invention as a starting point for further experimentation to try various conditions that maybe treated by

using homeopathic potencies of purified IGF-1. In addition, because there are no working examples provided describing the treatment of various physiological disorders, which use IGF-1, it would require an undue amount of experimentation to one of skill in the art to practice the claimed invention.

In addition, there is no guidance provided for the mechanism associated with the various physiological that are treatable using homeopathic potency of IGF-1 recited in the claims. While mechanism is not required, it can allow extrapolation of enablement to non-exemplified embodiments. Since applicant has not provided any working examples to teach the use of a preparation comprising homeopathic potency of purified IGF-1 suitable for oral administration for treating a subject experiencing a physiological disorder either *in vitro* or *in vivo*, it would require an undue amount of experimentation to one of skill in the art to practice the invention as claimed.

Given the breadth of claims 1, 2, 11, 13-28 and 30-33 in light of the unpredictability of the art as determined by the lack of working examples, the level of skill of the artisan, and the lack of guidance provided in the instant specification and the prior art of record, it would require undue experimentation for one of ordinary skill in the art to make and use the claimed invention.

8. No claims are allowable.

Contact Information

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jegatheesan Seharaseyon, Ph.D whose telephone number is 571-272-0892. The examiner can normally be reached on M-F: 8:30-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on 571-272-0961. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

JS
Art unit 1647,
February 20, 2007

CHRISTINE J. SAOUD
PRIMARY EXAMINER
Christine J. Saoud

Notice of References Cited	Application/Control No. 10/001,367	Applicant(s)/Patent Under Reexamination BREWITT, BARBARA A.	
	Examiner Jegatheesan Seharaseyon, Ph.D	Art Unit 1647	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,466,670	11-1995	Dunger et al.	514/12
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Pettit et al. The development of site-specific drug -delivery systems for protein and peptide biopharmaceuticals(1998), Trens in Biotech 16; 343-349.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals

Dean K. Pettit and Wayne R. Gombotz

The desire to deliver protein and peptide biopharmaceuticals conveniently and effectively has led to intense investigation of site-specific drug-delivery systems. Despite challenges, progress towards the convenient noninvasive delivery of proteins and peptides has been achieved through specific routes of administration. In addition, the delivery of proteins and peptides to specific sites of action has been utilized to lower the total delivered dose, to gain access to specific organs or body compartments and to concentrate a therapeutic dose at a specific site of pharmacological action.

A complete definition of 'site-specific' drug delivery needs to encompass both the route of administration and the site of action. Here, we define site-specific drug delivery as delivery through a specific site (i.e. the route of administration) as well as delivery to a specific site (i.e. the site of action). Various routes of administration have been investigated to deliver proteins and peptides, including pulmonary, oral, mucosal-membrane and transcutaneous[1–12]. Administration via these routes often requires delivery vehicles and/or permeability enhancers, which assist in transfer across the delivery site and into the systemic circulation. Proteins and peptides have also been delivered to specific sites of action such as organs, tissues, cells and molecular targets[13–39]. As with the route of administration, targeting a specific site of action often requires the aid of a delivery vehicle that relies on the specific properties of the protein or peptide to be delivered, as well as the unique properties of the tissue being targeted. In this article, we will summarize several examples of both the route of administration as a means to target systemic circulation and several site-specific drug-delivery systems designed to deliver proteins and peptides to specific tissues or sites of action. Examples of route-specific and site-specific drug delivery are too numerous to review completely and so we will merely provide examples to give the reader a sense of the breadth of approaches currently in use or under investigation. Although many examples of site-specific delivery systems for synthetic small-molecule drugs have been reported in the literature (some of which are commercially available), this review will focus on the site-specific delivery of proteins and peptides. An outline of the examples described in this review is shown in Table 1.

Delivering proteins and peptides through a specific site

As mentioned above, the goal of delivering proteins and peptides noninvasively has led to intense investigation; however, success in achieving this goal has been modest, with poor applicability to proteins and peptides in general. The challenges posed by proteins and peptides include their large molecular size, their electrical charge and relatively hydrophilic nature (which diminish membrane transport), and their relative instability in environments of extreme pH or proteolytic activity (such as the stomach and intestine). Permeation enhancers have frequently been used to enhance membrane transport of unmodified proteins and peptides (Fig. 1a).

Through the skin

Considerable effort has been given to the transdermal delivery of pharmaceutical products, but clinical applications have thus far been limited to non-protein drugs. The poor inherent permeability of skin to proteins and peptides has led to the investigation of chemical enhancers and the use of electricity (iontophoresis), but their efficacy has been limited by the large size and relatively low electrical charge of proteins. Some success has been reported in delivering peptides such as antiflammin 1 with the aid of electrical charge[1], and it has recently been reported that proteins as large as insulin (~6000 kDa), interferon γ (~17 000 kDa) and erythropoeitin (~48 000 kDa) could be delivered across the skin at therapeutic concentrations with the aid of low-frequency ultrasound[2]. Although these examples give encouragement to the transdermal delivery of proteins and peptides, it is doubtful that this route will provide a general approach to noninvasive delivery of these compounds.

Through the lungs

Inhalation therapy provides an attractive route of administration for the systemic delivery of peptide and protein pharmaceuticals[3,4]. Of the noninvasive routes for the delivery of proteins and peptides, the pulmonary route has provided the most encouraging data and has recently generated great interest from the biotechnology industry. Dry or liquid particles can be prepared and inhaled with the aid of dry-powder dispersers, liquid-aerosol generators or nebulizers. These devices produce particles that range in size from 1 μm to >10 μm. Although inhaled particles more than 10 μm in diameter are trapped in the nasal passages, throat, larynx and bronchial walls, those less than 5 μm in diameter may penetrate deeply into the alveoli of the lung[5].

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 0167 – 7799/98/$19.00. PII: S0167-7799(98)01186-X

Table 1. Some examples of site-specific drug delivery

Site targeted	Comments	Refs
Route of administration		
Transdermal	Assisted by iontophoresis or ultrasound	1,2
Pulmonary	Liquid and dry-powder aerosol delivery	3–5
Mucous membranes	Aerosol–mucin charge interactions	6,7
Oral/intestinal	Small particles, protein–carrier complexes	8–12
Systemic circulation		
Injection	Prolong or sustain circulation	13–19
Specific tissues or organs		
Tumors	Neovascularization markers are targeted	20
Lungs	Aerosol, liposomal delivery	16,21,22
Brain	Target the transferrin receptor	23–25
Intestines	Protect against proteolysis and acid hydrolysis	26
Eyes	Mucin charge interactions	27
Uterine horns	Form biodegradable gel *in situ*	28
Bones	Hydroxyapatite binds bone-promoting growth factor	29
Skin	Methylcellulose gels	30
Cellular/intracellular		
Macrophages	Small particles are phagocytosed	31,32
Tumor cells	Fusogenic liposomes to deliver intracellular toxins	33,34
Molecular targets		
Tumor antigens	Antibody–enzyme conjugates activate prodrugs	35
Fibrin/site of clot formation	Fusion proteins combine targeting with toxin	36,37
Carbohydrate receptors	Mannose and galactose used to target receptors	38,39

Once deposited deep in the lung, the alveoli provide a large surface area (80–140 m^2) for rapid transfer into the pulmonary circulation.

The routine pulmonary delivery of peptide and protein pharmaceuticals faces several challenges. Delivery-device issues must be addressed, and several devices are currently being tested and marketed, including nebulizers and liquid-aerosol generators (which deliver liquid aerosols), and dry-powder dispersers (which deliver lyophilized, jet-milled or spray-dried powder formulations). Protein-stability issues must also be overcome: dry-powder and liquid formulations may require buffers to maintain pH, surfactants such as Tween® to reduce protein aggregation and stabilizers such as carbohydrates (e.g. sucrose, trehalose) to prevent denaturation during prolonged storage. Also, although most proteins or peptides can eventually be delivered into the lungs with the appropriate formulation and device characteristics, the bioavailability of proteins (i.e. the amount of protein that actually crosses from the alveoli into the pulmonary circulation) is largely dependent on the physical characteristics of the delivered protein and is not the same for proteins and peptides in general. Despite the challenges facing pulmonary delivery, several proteins and peptides are currently under investigation for systemic delivery, including insulin, leuteinizing-hormone-releasing hormone (LHRH) analogs, granulocyte colony-stimulating factor (G-CSF) and human growth hormone (hGH)[4].

Through mucous membranes

The high vascularity and accessibility of the mucous membranes have made this tissue a potential route of administration for proteins and peptides. Mucous membranes that may allow systemic access include the buccal, nasal, vaginal and rectal membranes. The nasal route has been frequently investigated because of its convenience; large (>10 µm) aerosol particles or drops can be generated to deliver protein and peptide drugs through the nasal mucosa. Permeation enhancers and protease inhibitors usually need to be coadministered to achieve successful delivery by this route. Extensive research has been carried out with peptides and small proteins, and several products have reached the market place, including buserelin, desmopressin, oxytocin and calcitonin[6].

Calcitonin has also been delivered by the vaginal route following encapsulation into esterified hyaluronic acid (Hyaff™) microspheres[7] (Fig. 1b). This example demonstrates how the combination of physical properties of the delivery device and those at the site of delivery may be used to regulate delivery of the drug. The Hyaff™ microspheres adhered to the mucosal tissue by charge interactions, allowing slow degradation of the microspheres and thus releasing the calcitonin slowly. The microspheres also served to protect the calcitonin from proteolytic digestion while adhering to the mucosal tissue.

Oral – through the intestine

The potential convenience of oral delivery has led to considerable research in this area[8,9]. However, with the exception of cyclosporin (a cyclic peptide consisting of ten amino acids), very limited success has been achieved delivering peptides and proteins via this route. The primary problems encountered with the oral delivery of

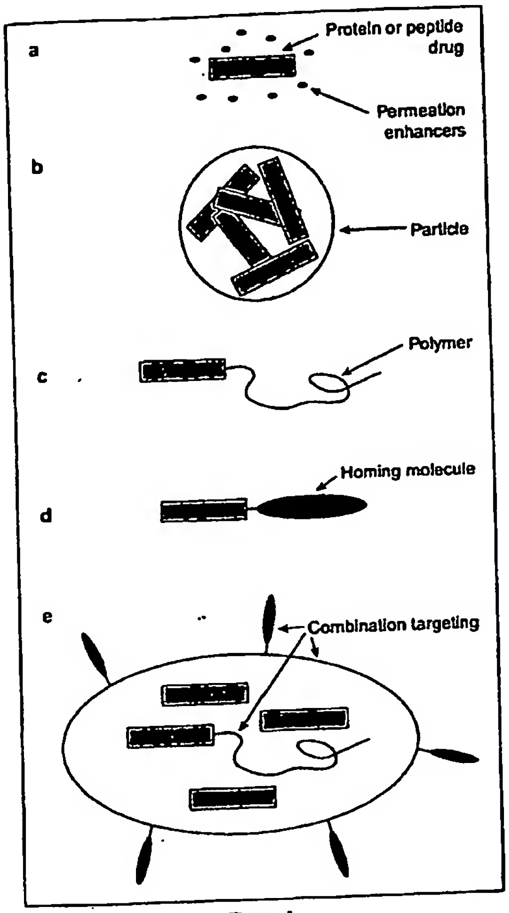

Figure 1

Site-specific protein- and peptide-drug delivery is often achieved by engineering molecular or macroscopic systems in order to target a specific route of administration or site of action. (a) A protein or peptide may be targeted to a site by taking advantage of such natural physical characteristics as molecular weight, net ionic charge, and molecular specificity. Permeation enhancers are often utilized to improve delivery through specific routes. (b) Encapsulation of proteins and peptides into particles such as aerosols, degradable microspheres, liposomes or other matrices takes advantage of macroscopic-size effects for targeting tissues and cells. Drug-carrying matrices may also be formed into sheets, tubes and other morphologies. (c) Modification of proteins and peptides with synthetic polymers, such as poly(ethylene glycol), or natural polymers, such as polysaccharides, may alter their physical characteristics and favor targeting to specific compartments, organs or other tissues within the body. (d) The linkage of proteins and peptides to homing molecules of molecular specificity provides a direct approach to target unique sites. (e) Various combinations of polymers, particles and homing molecules have also been used to deliver protein and peptides specifically.

protein pharmaceuticals include: (1) the poor intrinsic permeability across intestinal epithelium; (2) susceptibility to enzymatic attack; (3) rapid post-absorptive clearance; and (4) chemical instability. Despite these challenges, some interesting reports have suggested that the transport of peptides, and even larger proteins, may be feasible.

One possible route of passage across the intestinal lining is through the Peyer's patches, which are a key component in the lymphatic system of the intestines. Investigators have reported that small microspheres (≈100 nm) prepared from poly(lactide-*co*-glycolide) (PLGA) are preferentially taken up into Peyer's patches[10]; this observation may lead to the development of orally delivered protein and peptide vaccines. In another recent report, investigators have prepared microspheres from polyanhydrides that exhibited strong bioadhesive properties to the mucous lining of the intestines[11]. In this study, microspheres were found to traverse the intestinal lining and it was shown that insulin delivered with these microspheres was systemically active in rats. Finally, it has been demonstrated that coadministration of hGH with α-amino acids could significantly enhance the bioavailability of this protein; it has been suggested that a complex formed between hGH and the α-amino acid may be responsible for passage across the epithelial lining[12]. The mechanism of action in this example is unclear, but the observation has been repeated with other proteins that have been specifically paired with various other α-amino acids.

Targeting the systemic circulation

Once administered, many biopharmaceuticals carry out their intended pharmacological action in the systemic circulation. Novel developments in systemic administration involve prolonging the action of circulating proteins. In order to do this, these compounds must resist clearance by conventional mechanisms, including molecular filtration by the kidney (which selectively removes smaller molecules from the circulation in favor of larger molecules) and clearance by the reticuloendothelial system (RES) (which favors clearance of larger, hydrophobic particles over smaller, more hydrophilic ones). Systems that target the systemic circulation are generally characterized as 'passive' delivery systems (i.e. targeting occurs because of the body's natural responses to the physical characteristics of the drug or drug-delivery system).

Many proteins and peptides have been conjugated with polyethylene glycol (PEG) (Fig. 1c) in order to reduce renal clearance and enhance their systemic circulation half-lives[13]. PEG-conjugated proteins have been shown to increase the circulation half-lives of certain peptides and proteins greater than 50-fold[13,14], but PEGylation often leads to a reduction or alteration in the biological activity of protein pharmaceuticals, owing to steric interference of the conjugated PEG with biologically relevant regions of the protein[14]. Despite the challenges of producing biologically active PEG–protein conjugates, several PEGylated protein pharmaceuticals are currently progressing through clinical trials (e.g. PEG–α-interferon, PEG–G-CSF, PEG–interleukin-2, PEG–hemoglobin) and others are now commercially available (e.g. PEG–L-asparaginase, PEG–adenosine-deamidase).

Degradable polymer microspheres have also been designed to maintain the systemic levels of peptides such as LHRH[15], proteins such as growth hormone[16] (GH) and granulocyte–macrophage colony-stimulating factor (GM-CSF)[17], and vaccines such as staphylococcal

enterotoxin B (Ref. 18). Microspheres prepared from degradable polymers such as PLGA, polyanhydrides and others [in which proteins or peptides are trapped within a polymer (Fig. 1b)] have frequently been reported in the literature[19]. Following administration, these microspheres slowly release the encapsulated proteins or peptides; the polymer microspheres eventually degrade into biologically inert byproducts. Considerable commercial success has been achieved by the encapsulation of LHRH in PLGA microspheres[15].

Targeting specific tissues or organs

Many tissues possess specific physical or chemical characteristics such as elevated blood flow, selective membrane permeability or overall net charge, or perform their routine physiological function under extreme conditions such as low pH. These properties have been exploited for the development of site-specific drug-delivery systems.

Tumors

As a tissue, large tumors often differ from otherwise healthy tissue in a manner that can be exploited for site-specific drug-delivery techniques. Researchers have taken advantage of the fact that tumors often require a large blood supply and demand highly vascularized tissue to maintain their rapid rate of growth. New-blood-vessel formation provides a general target by which drugs may be directed to rapidly developing tumors. Investigators have reported the use of antibodies against tumor-derived vascular-endothelial cells (TEC) in order to target these rapidly developing blood vessels[20] (Fig. 1d); in these experiments, anti-TEC antibodies have been shown to suppress tumor growth significantly in rats that carried a fibrosarcoma. The appeal of such a targeting approach is that, unlike tumor antigens (which may not be easily accessible to antitumor antibodies), antigens on the surface of TECs are readily accessible by intravenous antibody injection.

Lungs

In addition to drug delivery through the lungs for systemic availability, as discussed above, drugs may also be targeted to the lungs for the local treatment of pulmonary disorders. One such protein used for local pulmonary therapy is DNase, which aids patients with cystic fibrosis by cleaving DNA released from leukocytes that have accumulated in the fluid of the lungs; the ability of the lungs to expel this fluid is enhanced and lung function improved following this treatment. Considerable work on DNase was necessary to deliver it by nebulization for local pulmonary delivery[21]. Other proteins under investigation for local delivery to the lungs include cyclosporin, interferon and α-1 antitrypsin[16].

As another example of targeting proteins to the lungs, a liposomal delivery system has been developed that targets superoxide dismutase (SOD) to the pulmonary epithelium[22]. Treatment with SOD prevents tissue damage to the lungs of patients undergoing long-term oxygen therapy by removing free oxygen radicals. In this study, SOD was encapsulated into liposomes (Fig. 1e) prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-oleoyl-2-oleoyl-*sn*-glycero-3-succinate (DOSG) and surfactant-protein A (SP-A); the SP-A was utilized for targeting liposomes to pulmonary epithelial cells, which express a high-affinity SP-A receptor. This formulation was found to enhance cell-associated SOD activity more than five-fold *in vitro*. Methods to deliver these and other types of liposomes to the lung via aerosol delivery have also been under investigation.

Brain

The delivery of peptides and proteins into the brain is particularly challenging because of the so-called 'blood–brain barrier', which limits the transfer of soluble drugs through the brain-capillary endothelial wall. Attempts to deliver peptides, proteins and other soluble drugs to the brain include methods that break down the blood–brain barrier (e.g. leukotrienes and hyperosmolar shock), but the loss of this barrier function may allow uncontrolled access of solutes into the brain. The direct injection of protein or peptide drugs into the brain is also undesirable because diffusion of these molecules is generally poor in parenchymal brain tissue.

Methods of site-specific delivery of peptide and protein drugs into the brain have taken advantage of a specific receptor-mediated pathway in the blood–brain barrier: transferrin receptors are abundant on the vascular endothelium of brain capillaries, and these receptors are internalized by the endothelial cells in a process designed to deliver iron to the brain. This allows the blood–brain barrier to be bypassed by using transferrin or transferrin-receptor antibodies as carriers of proteins such as antibodies[23] and neuropeptides[24]. In this embodiment, the targeting system (i.e. transferrin or transferrin-receptor antibodies) is conjugated or fused by recombinant-DNA methods to protein and peptide drugs to produce the drug-targeting system (Fig. 1d). A number of drug-delivery vectors designed to cross the blood–brain barrier have recently been reviewed[25].

Intestines

Epithelial cells lining the small intestine proliferate rapidly and are particularly sensitive to cytotoxic drugs such as chemotherapeutics, which target rapidly diving cells. Investigators have utilized transforming growth factor β_1 (TGF-β_1) to inhibit intestinal-epithelial-cell proliferation, thereby sparing these cells from the toxic effects of chemotherapeutic agents[26]. In this research, TGF-β_1 was trapped within calcium-alginate microbeads by charge interaction between the TGF-β_1 and the calcium alginate. On exposure to low-pH conditions (i.e. as found in the stomach), the alginate precipitated, causing the beads to shrink and preventing the release of TGF-β_1, protecting the TGF-β_1 from the harmful effects of the low-pH environment. Following passage into the small intestine, the pH increased, allowing complete and rapid release of the TGF-β_1. Histological evaluation of rats administered TGF-β_1–alginate beads indicated that the TGF-β_1 was indeed delivered to the intestinal epithelial cells and inhibited cell proliferation[26].

Eyes

The delivery of biopharmaceuticals to the surface of the eye is complicated by the normal processes of blinking, tearing and drainage from the eye, which rapidly remove drugs that are administered without a delivery vehicle. Epidermal growth factor (EGF) has been

studied as a factor for the treatment of corneal wounds and conditions of abnormal corneal-epithelial wound healing. The delivery of soluble EGF has been shown to be ineffective in many of these conditions, even at eight doses per day. To achieve constant levels of EGF in the tear fluid, a combination of EGF with poly(acrylic acid) gels has been found to be successful for the treatment of corneal epithelial wounds[27]. Poly(acrylic acid) is known to be a bioadhesive polymer, owing to its negative ionic charge, and has been shown to interact with and bind to conjunctival cells. In this case, the poly(acrylic acid) gels were retained in the eyes over a period of 8 h and EGF concentrations in the tear fluid were maintained at constant levels throughout this period. The rate of epithelial-cell healing was found to increase in a dose-dependent fashion when EGF was combined with the poly(acrylic acid) gels. This example demonstrates the ability to use the charge characteristics of the delivery device to target a drug to a tissue of complementary charge.

Uterine horns

Proteins have been applied directly to tissues such as the uterine horns with the aid of biodegradable hydrogels in an attempt to prevent post-surgical adhesions[28]. Tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) was coapplied with poly(ethylene glycol) (8000 Da) that had been extended with an average of five lactic-acid residues on each end and further capped at each end with a photoreactive acrylate unit; polymerization of the biodegradable hydrogel occurred by exposure of the polymer mixture to ultraviolet light following application of the solution and protein to the uterine horn. Post-surgical adhesions were significantly inhibited by the combination of tPA or uPA with the biodegradable hydrogel.

This example demonstrates proteins being targeted by direct application to the tissue with the aid of a polymer or device designed to hold the protein at the site of action. Other examples of this principle include the application of TGF-β_1 to the surface of titanium hip implants coated with hydroxyapatite and tricalcium phosphate to enhance bone growth[29], and the application of growth factors such as acidic fibroblast growth factor (aFGF) directly into topical wounds with the aid of methylcellulose gels[30].

Cell-targeted drug delivery

Proteins and peptides are often targeted into cells. The specific properties and functions of cells, such as the ability of macrophages to phagocytose particles of a certain size, have been utilized to develop these drug-targeting systems.

Macrophages act as mediators of the foreign-body response by ingesting foreign bodies and presenting foreign antigens to other cells that regulate the immune system; consequently, macrophages have been used to deliver antigens or vaccine adjuvants in a site-specific manner. In one example, human serum albumin was conjugated to the surface of poly(4-aminostyrene) microspheres[31]. These microspheres (1 μm diameter) were shown to be taken up by macrophages following subcutaneous injection into mice, and evaluation of blood from these animals demonstrated strong antigen-specific peripheral-blood monocytic cell (PBMC) responses, as well as high antibody titers. It was concluded that surface-conjugated microspheres could be effectively targeted to macrophages without the aid of a vaccine adjuvant to elicit an immune response.

In a second example, a model protein antigen [human γ globulin (HGG)] was encapsulated in gelatin microspheres crosslinked with glutaraldehyde; these microspheres were intended to be taken up by macrophages and deliver their contents intracellularly. Anti-HGG-antibody titers were measured following the subcutaneous administration of the microspheres in mice, and the production of specific antibodies was found to be related to the concentration of the glutaraldehyde crosslinking reagent. At a low glutaraldehyde concentration, the microspheres were extensively swollen, leading to an increase in the size of hydrated microspheres and a decrease in their macrophage phagocytosis; at high glutaraldehyde concentration, the release rate of the antigen from the microspheres was low. An intermediate glutaraldehyde concentration was thus found to maximize the antibody titer[32].

Cells have also been targeted by fusogenic liposomes, which fuse with a cell membrane and direct their contents into the cell. This application may find particular application in the delivery of RNA and DNA, but protein delivery has also been successfully demonstrated. In an early report, fusogenic liposomes were prepared from phosphatidylcholine, phosphatidylserine and cholesterol in the presence of fragment A of diphtheria toxin (DTA)[33]; DTA lacks the normal cell-binding domain and can exhibit cytotoxicity only when delivered intracellularly. The addition of these fusogenic DTA-liposomes to cell cultures clearly demonstrated cytotoxicity, whereas unencapsulated DTA did not, indicating that DTA could be successfully administered into the cells by the fusogenic liposomes. This concept was more recently applied to the treatment of tumors in the peritoneal cavity[34], in which DTA-liposomes were shown to kill sarcoma-180 cells effectively both *in vitro* and *in vivo*.

Molecular targets for drug delivery

Molecular targets provide the most specific form of protein- and peptide-drug targeting. In one sense, many protein and peptide drugs themselves already target a specific receptor or ligand. For example, insulin will circulate systemically and either be cleared from the body or bind specifically to an insulin receptor. However, there are other examples in which the function of a protein or peptide drug is enhanced when it can be targeted to a specific site of action. Molecular targets provide a means for 'active' drug delivery; that is, the site to which the drug is ultimately delivered depends on the molecular specificity of the targeting agent rather than the physical characteristics of the drug or drug-delivery system.

Antibody conjugates and antibody fusion proteins have long been touted as potential targeting agents to deliver proteins, peptides and other drugs to specific sites of action, such as tumors. In one area of investigation, antibodies against tumor antigens have been conjugated to enzymes capable of converting a soluble prodrug such as doxorubicin phosphate into an active drug at the site of action[35]. In this technique, the antibody is administered prior to the drug so that

Table 2. Parameters engineered to control site-specific delivery of proteins and peptides

Engineering parameters involved	Some examples	Refs
Properties of proteins and peptides		
Molecular weight	Low molecular weight or cyclic peptides are more readily transported across biological membranes. Conjugation of PEG to peptides and proteins enhances circulation half-lives.	6,13,14
Ionic charge	Cationic polymer binds anionic protein or peptide to anionic tissue.	27
Molecular specificity	Transferrin receptors transport proteins and peptides across the blood–brain barrier; chimeric fusion proteins combine the pharmacological activity of proteins and peptides with targeting moiety.	23–25,36,37
Properties of delivery systems		
Particle size	Degradable microspheres less than 5 μm diameter are phagocytosed by macrophages; aerosol particles from 1 to 5 μm are targeted to deep alveoli in the lungs.	5,31
Biodegradability	Degradable PLGA microspheres release proteins and peptides slowly, enhancing systemic circulation.	15–19
Tissue adhesiveness	*In situ* polymerizing PEG–acrylate adheres to uterine horns.	28
Hydrophobicity	Fusogenic liposomes fuse with cell membrane to deliver drugs intracellularly.	33,34
Properties of tissues and cells		
Membrane permeability	Permeability enhancers improve the transport of proteins and peptides.	6
Local pH	Alginate microspheres collapse under the acidic conditions of the stomach and solubilize under the neutral conditions of upper gastrointestinal tract	26
Antigen expression	Overexpression of tumor antigens allows targeting with antibodies.	35

unbound antibody can be cleared from the circulation and the toxic effect of the drug limited to those sites at which the antibody, and thus the enzyme, has bound to the tumor antigen.

Many chimeric proteins have been constructed from molecules of two different functionalities fused together using recombinant methods. Immunotoxins are an example of chimeric fusion proteins in which a cytotoxic subunit, such as *Pseudomonas* exotoxin (PE), is fused with a targeting molecule, such as transforming growth factor α (TGFα)[36]. The TGFα binds to cells that express the TGFα receptor; the PE carries out its function and kills the cell to which the molecule has bound. This particular fusion protein was prepared for the treatment of non-small-cell lung carcinoma, which overexpresses the TGFα receptor on the cell surface. Another example of a chimeric protein used for drug targeting is the fusion of tPA with an antifibrin antibody[37]; the thrombolytic potential of tPA may be enhanced by directing the molecule to the site of action, specifically a fibrin surface, where a blood clot may be initiated.

The ability of carbohydrates to target cells or tissues with carbohydrate receptors has also been investigated. Notably, the asialoglycoprotein receptor on hepatocytes and the mannose receptor on macrophages specifically recognize compounds with terminal galactose and mannose residues, respectively, and are widely used in the selective delivery of drugs to these cells[38,39]. In this system, the carbohydrate may be viewed as the targeting molecule, which has been conjugated to a protein or peptide drug (Fig. 1c).

Conclusions and future outlook

Techniques for site-specific protein- and peptide-drug delivery must take into consideration the physical and chemical characteristics of both the protein to be delivered and the site to be targeted. No generalities can be made in this regard. In many instances, a synthetic polymer, device or carrier system is introduced with the appropriate properties to target a protein to a specific site within the body. Some of the characteristics of the proteins, peptides or delivery systems themselves that have been exploited to influence targeting include molecular weight or particle size, ionic charge, hydrophobicity, membrane or device permeability, susceptibility to biodegradation, and molecular specificity. Each of these properties has been successfully utilized to achieve site-specific delivery of proteins and peptides under specific conditions (Table 2).

Protein- and peptide-drug-delivery systems are perceived by many as second-generation products designed to improve convenience and compliance, reduce the dosing frequency or reduce the total dose required to achieve the pharmacological effect of an established product. For the few drug-delivery systems that are currently marketed for protein and peptide drug delivery, these goals have been achieved successfully. In the future, targeted drug-delivery systems may also prove particularly valuable to enable the use of a particular drug that would otherwise be ineffective or even toxic if delivered systemically [e.g. neural growth factors (which need to cross the blood–brain barrier) or vaccines (which need to be taken up by antigen-presenting cells)]. At the current pace of gene cloning

and recombinant-protein production within the biopharmaceutical industry, many more site-specific drug-delivery products will be clinically investigated and implemented in the near future.

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Some caveats for bioengineering terpenoid metabolism in plants

David McCaskill and Rodney Croteau

The engineering of terpenoid formation in plants, although highly appealing from a biotechnological viewpoint, is particularly challenging because of the myriad of terpenoids produced from a single intermediate (isopentenyl diphosphate) and the complex organization and subtle regulatory features of the biosynthetic pathways. This article surveys many of the biochemical issues that must be appreciated before attempting to develop rational strategies for the bioengineering of terpenoid biosynthesis.

Terpenoids are a chemically diverse family of compounds that are found throughout Nature and are essential for the normal growth, development and survival of every living organism. Terpenoids play essential roles in maintaining membrane fluidity (sterols), electron transport (ubiquinone, menaquinone and plastoquinone), glycosylation of proteins (dolichol) and the regulation of cellular development (hormones and terpenoid-modified regulatory proteins). The field of terpenoid biosynthesis is currently very active, with long-standing models undergoing fundamental reevaluation and revision. The basic classification of the various terpenoid families and the enzymology and regulation of the acetate–mevalonate pathway, prenyl transferases and terpenoid synthases have all been recently reviewed[1–3]; this article will focus on recent developments in the enzymology and regulation of terpenoid biosynthesis in plants, with emphasis on the implications of these current developments for the bioengineering of terpenoid formation. An appreciation of the subtle complexities of the regulation of terpenoid biosynthesis is necessary to avoid being led astray in our attempts to bioengineer the production of specific terpenoids in plants.

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